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Non-invasive monitoring of 21-hydroxylase deficiency

Measurement of salivary adrenal-specific androgens as biomarkers of therapy control in 21-hydroxylase deficiency

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Abbreviations

11KT: 11-ketotestosterone

11OHA4: 11-hydroxyandrostenedione

17OHP: 17-hydroxyprogesterone

21OHD: 21hydroxylase deficiency

A4: androstenedione

ACTH: andrenocorticotropic hormone

CAH: congenital adrenal hyperplasia

CYP: children and young people

CYP11B1: 11-hydroxylase

DHT: dihydrotestosterone

GC: glucocorticoid

HSD11B2: hydroxysteroid 11-beta dehydrogenase 2

ICH GCP: International Conference for Harmonization of Good Clinical Practice

IRAS: Integrated Research Application System

LC-MS/MS: liquid chromatography tandem mass spectrometry

PCOS: polycystic ovary syndrome

REC: Research Ethics Committee

T: testosterone

Background: Monitoring of hormonal control represents a key part of the management in congenital adrenal hyperplasia (CAH). Monitoring strategies remain suboptimal as they rely on frequent blood tests and are not specific for adrenal-derived hormones. Recent evidence suggests a crucial role of adrenal-specific 11-oxygenated-C19 androgens in the pathogenesis of CAH.
Objective: To establish the correlation between plasma and salivary adrenal-specific androgens in CAH as a novel non-invasive monitoring strategy.
Design: This prospective cross-sectional study recruited patients between 2015-2018.
Setting: Multi-center study including 13 tertiary centers in the UK.
Participants: Seventy-eight children with CAH and sixty-two matched healthy controls.
Methods: Using liquid-chromatography tandem mass spectrometry, plasma and salivary concentrations of five steroids were measured: 17-hydroxyprogesterone, androstenedione, testosterone, 11-hydroxyandrostenedione and 11-ketotestosterone. The correlation between plasma and salivary steroids was analyzed to assess their use in clinical practice.
Results: Strong correlations between plasma and salivary steroid concentrations in patients with CAH were detected: 17-hydroxyprogesterone (r_s=0.871, p<0.001), androstenedione (r_s=0.931, p<0.001), testosterone (r_s=0.867, p<0.001), 11-hydroxyandrostenedione (r_s=0.876, p<0.001), 11-ketotestosterone (r_s=0.944, p<0.001). These results were consistent for patient subgroups based on gender and age. Analyzing patient subgroups based on 17-hydroxyprogesterone concentrations established clear correlations between plasma and salivary concentrations of the adrenal specific androgen 11-ketotestosterone.
Conclusions: The present study identified tight correlations between plasma and saliva for the adrenal-derived 11oxygenated-C19 androgen 11-ketotestosterone, as well as 17-hydroxyprogesterone and androstenedione, which are widely used for monitoring treatment in CAH. This novel combination of steroid hormones will serve as an improved non-invasive salivary test for disease monitoring of patients with CAH.

Plasma and salivary concentrations of adrenal-specific 11oxygenated-C19 androgens closely correlate and are promising biomarkers for non-invasive therapy monitoring in congenital adrenal hyperplasia. .

Introduction

Steroid 21-hydroxylase deficiency (21OHD) represents the commonest form of congenital adrenal hyperplasia (CAH) and occurs in about 1 in 10,000 to 1 in 15,000 live births [1]. 21OHD results in impaired cortisol synthesis and therefore absence of the negative feedback towards the anterior pituitary and hypothalamus. Consequently, excessive ACTH secretion leads to adrenal hyperplasia and further androgen excess. In addition, two-thirds of patients suffer from clinically apparent mineralocorticoid deficiency resulting in renal salt loss. Thus, management of CAH entails life-long glucocorticoid replacement, as well as mineralocorticoid replacement in patients with additional salt loss. Treatment with glucocorticoids is not only required to replace deficient cortisol but also to reduce the ACTH drive and subsequent androgen excess [1, 2]. Meeting the balance between under-treatment leading to androgen excess and over-treatment leading to glucocorticoids over-exposure remains challenging. Thus, overall, CAH is associated with significantly increased mortality and morbidity [3].

Monitoring of hormonal control represents one of the most important cornerstones of clinical care provision for individuals with CAH [3]. Different approaches and combinations of steroid hormones are currently used. However, the majority of clinicians rely on the

measurement of 17-hydroxyprogesterone, androstenedione and testosterone concentrations in plasma at single or multiple time points [4]. Blood tests are invasive and distressing for children and young people, as accurate monitoring requires frequent measurements adding up to approximately 50 to 70 blood tests from birth to the age of 18 years. Additionally, some of the steroid hormones routinely measured are both derived from the gonad and adrenal, which makes interpretation of results from puberty onwards challenging.

In recent years, analytical methods in steroid hormone biochemistry have remarkably advanced allowing for more reliable measurement of hormonal concentrations in saliva using liquid chromatography tandem mass spectrometry (LC-MS/MS) [5]. LC-MS/MS was validated for measuring saliva concentrations for a number of steroid hormones including cortisol, cortisone, testosterone, androstenedione, dehydroepiandrosterone and 17-hydroxyprogesterone [5-7]. Studies exploring correlations between saliva and serum concentrations for these hormones showed reliable correlations [8-10]. In particular, it has been suggested that salivary androstenedione and 17-hydroxyprogesterone are of clinical use in monitoring therapy in 21OHD [9, 11, 12].

Recent evidence indicates a key role of adrenal-derived 11-oxygenated-C19 androgens in the pathogenesis of CAH [13, 14] (**Supplementary Figure 1**) [15]. These 11-oxygenated-C19 androgens derive from the adrenal gland and require 11-hydroxylation by 11-hydroxylase (CYP11B1) as well as 11-dehydrogenation by 11-hydroxysteroid dehydrogenase type 2 (HSD11B2). Some of these androgens, such as 11-hydroxyandrostenedione, are weak hormones serving as precursors for more potent androgens. However, 11-hydroxytestosterone and 11-ketotestosterone have strong androgenic activity of a similar magnitude to that of dihydrotestosterone (DHT) [16]. A recent study suggested that patients with 21OHD have significantly higher concentrations of 11-oxygenated-C19 androgens than healthy controls [13]. Furthermore, 11-hydroxyandrostenedione and 11-ketotestosterone appear to correlate with adrenal volume in patients with CAH [17]. Consequently, it was suggested that they might have clinical value as biomarkers of androgen excess in patients with CAH due to 21OHD [18].

The present study was designed to explore the possibility of using the measurement of adrenal-specific androgens in saliva as effective non-invasive methods of monitoring therapy control in patients with 21OHD. Therefore, we aimed to establish the correlation between plasma and salivary concentrations for adrenal-specific androgens that are relevant to monitoring disease control in 21OHD.

Materials and Methods

Study design and participants

The study was conducted in compliance with the NHS Research Ethics Committee (REC reference 15/YH/0537), the International Conference for Harmonization of Good Clinical Practice (ICH GCP) and the Research Governance Framework for Health and Social Care (2nd Edition).

The participants included patients with CAH aged between 8 and 18 years, from across the United Kingdom. Patients were recruited as part of a prospective cross-sectional, multi-center study of the health status of children and young people with CAH (CAH-UK, IRAS ID 191301). Patients were recruited through 13 participating UK regional centers (**Supplementary Table 1**) [15] providing care and long-term follow-up for CAH. The following inclusion criteria were used in the recruitment process: i) patients with known CAH due to 21-hydroxylase deficiency confirmed by hormonal and/or genetic testing, ii) age between 8 and 18 years and iii) capacity to assent/consent and provide signed and dated informed consent. The only exclusion criterion consisted of pregnancy. A control group of healthy children and young people (CYP) matched for age, sex and BMI was recruited by

local advertisement placed with the associated universities, NHS Trusts and GP surgeries. The following inclusion criteria were used for the control group: i) age between 8 and 18 years and ii) capacity to assent/consent and provide signed and dated informed consent. Exclusion criteria consisted of the following: i) past or present history of endocrinopathy (all stages), ii) type 1 or 2 diabetes, insulin resistance, iii) known condition of lipid/cholesterol metabolism, iv) presence of any psychiatric disorder, v) current or past use of psychiatric medication, vi) glucocorticoid use within the last 6 months, vii) diagnosed learning difficulties and/or full-scale IQ<70, viii) medication known to affect steroid metabolism and ix) pregnancy.

Paired blood and saliva samples were collected both from patients and controls. Sample collection was performed in the morning between 8:00 and 9:00 a.m. using standardized collection methods (sample collection protocols available as **Supplementary Information** [15]). For patients with CAH the samples were collected after the morning glucocorticoid dose. The samples were obtained locally in the participating centers, then sent for centralized steroid hormone analysis by LC-MS/MS.

Steroid hormone analysis

The samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS), measuring plasma and salivary concentrations for three parameters commonly employed in monitoring CAH control and two 11-oxygenated C19 androgens: 17-hydroxyprogesterone (17OHP), androstenedione (A4), testosterone (T), 11-hydroxyandrostenedione (11OHA4) and 11-ketotestosterone (11KT). In addition, concentrations of plasma cortisol, salivary cortisol and salivary cortisone were also measured.

Salivary Methods:

The LC-MS/MS assay used for the measurement of salivary hormones concentrations and its validation were recently described in another paper [19]. Samples (300 µL unstimulated whole saliva) and internal standard (20 µL) were prepared by supported liquid extraction (SLE) with dichloromethane and were reconstituted in 40% methanol. The internal standard mixture contained [$^{13}C_3$]-(2,3,4)–17-alpha-hydroxyprogesterone, D7-(2,2,4,6,6,16,16)-4-androstene-3,17-dione, [$^{13}C_3$]-(2,3,4)-testosterone, D7-2,2,4,6,6,16,16-4-Androsten-11β-ol-3,17-dione, D3-16,16,17-11-ketotestosterone. After on-line solid phase extraction with C18 cartridges, liquid chromatography was performed on a C8 column using a water/methanol gradient containing 0.1% formic acid and 2 mmol/L ammonium acetate. A Waters TQ-S mass spectrometer operated in positive ion mode was used for quantification. Total run time was 6.4 minutes. The full validation of the assay has been published previously [19]. In brief, recovery was between 89% and 109%, ion suppression between 86% and 105% for all analytes. Intra- and inter-assay comparisons showed a coefficient of variation (CV) <10% and the bias between measured and nominal concentration was between -8% and 10%. Interference with a large set of natural and synthetic steroids was excluded. The lower limits of quantification, defined as the lowest concentrations with a CV and a bias of <20% for 10 replicates, was 12.5 pmol/L for 17OHP, 6.25 pmol/L for A4 , 5 pmol/L for T , 50 pmol/L for 11OHA4 , 5 pmol/L for 11KT . Post-extraction stability was tested for an overnight period at 10 °C (17OHP, A4 and T) or at 4 °C (11OHA4 and 11KT). The bias between the direct and post-storage analysis was between -8.4 and 8.8% for 17OHP, -14.9 and 4.9% for A4, -30.6 and 19.9% for T, -8.8 and 4.8% for 11OHA4 and -8.3 and -0.5% for 11KT. Carry-over was 0.15% for 17OHP, 0.62% for A4, 0.65% for T, 0.075% for 11OHA4 and 0.036% for 11KT [19].

Plasma Methods:

Plasma samples (50 µL) and internal standard were prepared by SLE with methyl-tert-butyl-ether and were reconstituted in 55% methanol. The internal standard mixture was similar to

the saliva method. Liquid chromatography was performed on a Waters Acquity UPLC® HSS T3 1.8 µm, 50 x 2.1 mm analytical column using a water/methanol gradient containing 0.1% formic acid and 2 mmol/L ammonium acetate. A Waters TQ-S mass spectrometer operated in positive ion mode was used for quantification. Total run time was 4 min per sample. For all analytes, recovery was between 96% and 109%, matrix effects were negligible (<10%). Intra- and inter-assay comparisons showed a CV <8% and the bias between measured and nominal concentration was < 3% for 11OHA4 and 11KT and <7% for T, A4 and 17OHP. Interference with a large set of natural and synthetic steroids was excluded. The limit of quantitation was 0.1 nmol/L for T, A4, 11KT, 11OHA4 and 0.2 nmol/L for 17OHP. A rapid assay was conducted for plasma cortisol analysis as previously described [20].

Statistical analysis

Data was tested for normality using the Shapiro-Wilk test and non-parametric tests were employed for data that was not normally distributed. The Chi-squared test was used to compare patients and controls in relation to age groups, sex and pubertal stage. Steroid hormones concentrations between groups were compared using the Mann Whitney U test. To explore the relationship between plasma and salivary hormone concentrations we used Spearman correlations. A p value of < 0.05 was considered statistically significant. For both patients and controls the plasma-saliva pairs were excluded from the analysis if hormone concentrations were undetectable or could not be reported due to sample inadequacy. Statistical analysis and computation were carried out using SPSS Statistics Software Version 25 and GraphPad Prism 7.

Results

Characteristics of participants

The studied cohort consisted of 78 patients (35 males, 43 females) and 62 controls (26 males, 36 females). The median for age in the patient group was 12.8 years, interquartile range 10.4 to 15.6 years; for controls the median for age was 13.3 years, interquartile range 10.5 to 16.3 years. The patients were statistically similar to the controls with regards to gender and age distribution ($X^2(1, N=140) =0.121, p=0.72$ for gender, $X^2(1, N=140) =0.025, p=0.87$ for age), however, as expected, there were significant differences between the two groups in relation to pubertal status, with more CAH patients presenting advanced pubertal stage compared to controls ($X^2(1, N=140) =11.81, p=0.008$) (**Table 1**).

In the Patients group, 13 saliva samples (16.6%) were reported as insufficient or unsuitable. In the Controls group, four (6.4%) saliva samples were insufficient.

With regards to the glucocorticoid treatment, 72 of the patients were treated with hydrocortisone, with daily doses ranging from 4 to 27 mg/m^2 per day, six patients were treated with prednisolone with daily doses between 3 to 4 mg/m^2 per day (hydrocortisone equivalent of 15-20 mg/m^2 per day).

There was wide variability in the time elapsed from the administration of the glucocorticoid dose and the collection of the blood and saliva samples, ranging from 0 to 15 hours 15 minutes, with a median of 2 hours 29 minutes. For the majority of patients treated with hydrocortisone (41.4%) samples were collected between 2 and 3 hours after the glucocorticoid dose, with samples collected within 1 hour for 5.7% patients, 1 to 2 hours for 25.7%, 3 to 4 hours for 18.5%; for the rest of the patients (8.7%) samples were collected after 5 hours from the last dose of hydrocortisone.

Plasma and salivary hormone concentrations

Plasma and salivary hormone concentrations were significantly higher in patients when compared to controls (p<0.001) for four of the measured steroid hormones (androstenedione, 17-hydroxyprogesterone, 11-hydroxyandrostenedione and 11-ketotestosterone). Significant

differences were maintained for these hormones when subgroups of participants were compared based on gender and age. No significant differences for plasma and salivary testosterone were found when comparing patients and controls ($p=0.143$ for plasma, $p=0.681$ for saliva). However, when analyzing differences between subgroups based on age and gender, plasma testosterone was significantly higher in female patients ($p<0.001$) and patients younger than 12 years ($p=0.008$) in comparison to matched controls. By contrast, in male participants significantly higher plasma testosterone concentrations were found in controls than in patients ($p=0.01$). In pubertal boys plasma testosterone was significantly higher in controls compared to patients ($p=0.001$) (**Figure 1, Supplementary Tables 2 and 3**) [15].

Correlations between plasma and saliva steroid concentrations in patients with CAH

In patients with CAH due to 21OHD, strong correlations were detected between plasma and salivary steroid concentrations for all the five hormones analyzed. The strongest correlations were found for androstenedione ($r_s=0.931$, $p<0.001$) and 11-ketotestosterone ($r_s=0.944$, $p<0.001$). Weaker correlations between plasma and salivary hormone concentrations were detected for testosterone ($r_s=0.867$, $p<0.001$), 17-hydroxyprogesterone ($r_s=0.871$, $p<0.001$) and 11-hydroxyandrostenedione ($r_s=0.876$, $p<0.001$) (**Figure 2**). We obtained very similar results when investigating partial correlations between plasma and salivary steroid concentrations, controlling for time elapsed from glucocorticoid dose to sample collection and for glucocorticoid dose for body surface area (BSA): 17-hydroxyprogesterone ($r_s=0.809$, $p<0.001$), androstenedione ($r_s=0.909$, $p<0.001$), testosterone ($r_s=0.897$, $p<0.001$), 11-hydroxyandrostenedione ($r_s=0.875$, $p<0.001$) and 11-ketotestosterone ($r_s=0.915$, $p<0.001$) (**Supplementary Table 4**) [15]. In addition, strong correlations were observed in 21OHD patients when analyzing subgroups based on gender and age. In patients younger than 12 years of age, the strongest relationship between plasma and saliva was found for 17-hydroxyprogesterone ($r_s=0.923$, $p<0.001$), whereas in patients aged 12 to 18 years it was 11-ketotestosterone ($r_s=0.962$, $p<0.001$) that showed the best correlation between plasma and saliva. In male patients, the strongest correlation was noted for androstenedione and 11-ketotestosterone ($r_s=0.932$, $p<0.001$ for both hormones), while in girls the correlation found for 11-ketotestosterone ($r_s=0.940$, $p<0.001$) was slightly superior to androstenedione ($r_s=0.918$, $p<0.001$). For all subgroups of patients, the weakest correlation between plasma and saliva was detected for testosterone, with the exception of female patients where the correlation between plasma and saliva for testosterone was comparable to that of androstenedione (**Supplementary Table 5**) [15].

Acknowledging the markedly elevated concentrations of plasma 17-hydroxyprogesterone that were recorded in the patient group, we analyzed correlations between plasma and salivary steroid hormones separately for the 40 patients found to have 17-hydroxyprogesterone concentrations within the clinically relevant range (0 to 60 nmol/l). We found significant correlations for all the five steroid hormones, albeit weaker compared to the ones obtained when including all patients in the analysis: 17-hydroxyprogesterone ($r_s=0.768$, $p<0.001$), androstenedione ($r_s=0.896$, $p<0.001$), testosterone ($r_s=0.729$, $p<0.001$), 11-hydroxyandrostenedione ($r_s=0.770$, $p<0.001$), 11-ketotestosterone ($r_s=0.896$, $p<0.001$) (**Supplementary Figure 2**) [15]. Importantly, 11-ketotestosterone maintained the highest correlation between plasma and saliva of all the assessed steroid hormones.

Correlations between plasma and saliva steroid concentrations in the control group

In the control group, correlations between plasma and salivary steroids were generally weaker in comparison to the patient group: 17-hydroxyprogesterone ($r_s=0.641$, $p<0.001$), androstenedione ($r_s=0.925$, $p<0.001$), testosterone ($r_s=0.787$, $p<0.001$), 11-hydroxyandrostenedione ($r_s=0.828$, $p<0.001$), 11-ketotestosterone ($r_s=0.842$, $p<0.001$)

(**Supplementary Figure 3**) [15]. This may be explained by the fact that both plasma and salivary steroid concentrations were significantly lower in controls compared to patients (**Supplementary Tables 2 and 3**) [15]. When analyzing control subgroups we did not find a correlation between plasma and saliva testosterone in girls, while in children under 12 years we did not find correlations for 17-hydroxyprogesterone and testosterone (**Supplementary Table 6**) [15].

Cortisol and cortisone concentrations

In CAH patients plasma cortisol concentrations ranged from 14.7 to 827.3 nmol/l, with a median of 287.3 nmol/l, saliva cortisol ranged from 0.5 to 2410 nmol/l with a median 15.7 nmol/l and saliva cortisone ranged from 0.8 to 134 nmol/l with a median of 24.6 nmol/l. In 13 patients salivary cortisol exceeded plasma cortisol concentrations. For these samples, the collection time in relation to the previous hydrocortisone dose ranged from 90 to 205 minutes, which was similar to all other collected samples. The control group was statistically similar to patients with regards to plasma cortisol and salivary cortisone, while salivary cortisol concentrations were significantly lower in controls ($p < 0.001$) (**Supplementary Table 7 and Supplementary Figure 4**) [15]. We obtained similar results when limiting the analysis to patients treated with hydrocortisone (n=72). As expected, we found negative correlation between the time elapsed from hydrocortisone dose to sample collection and plasma cortisol ($r_s = -0.328$, $p=0.005$), salivary cortisol ($r_s = -0.282$, $p=0.027$) and salivary cortisone ($r_s = -0.262$, $p=0.031$). Plasma cortisol concentrations correlated well with salivary cortisone ($r_s=0.701$, $p<0.001$) but not with salivary cortisol. However, after excluding samples where salivary cortisol exceeded plasma cortisol from the analysis, we found a weak correlation between plasma and salivary cortisol ($r_s= 0.468$, $p=0.002$). By comparison, in healthy controls plasma cortisol correlated well with both salivary cortisone ($r_s=0.672$, $p<0.001$) and salivary cortisol ($r_s=0.677$, $p<0.001$). In patients with CAH, no correlation was detected for plasma or saliva between either cortisol or cortisone and any of the five adrenal steroids measured (**Supplementary Figure 5**) [15]. This was still maintained after excluding patients with suppressed 17-hydroprogesterone concentrations and controlling for the collection time. After excluding patient samples with a salivary cortisol greater than plasma cortisol concentrations from the analysis, we detected weak negative correlations between plasma testosterone and plasma cortisol ($r_s = -0.270$, $p=0.044$), salivary cortisol ($r_s = -0.305$, $p=0.049$) and salivary cortisone ($r_s = -0.305$, $p=0.047$), plasma androstenedione and salivary cortisol ($r_s = -0.318$, $p=0.043$). No additional significant relationship was found between cortisol or cortisone and any of the other androgens. By contrast, in healthy controls we found that plasma cortisol, salivary cortisol, salivary cortisone and the five adrenal steroids correlated in plasma and saliva, especially in the case of 11-hydroxyandrostenedione (**Supplementary Tables 8 and 9**) [15].

Patient classification in relation to plasma 17-hydroxyprogesterone concentrations

We classified patients according to plasma 17-hydroxyprogesterone concentration, defining concentrations within the range of 12 – 36 nmol/l as “normal’, less than 12 nmol/l as “low” and above 36 nmol/l as “high”. Only 18 (23.1%) patients were found to have 17-hydroxyprogesterone within the target range, 17-hydroxyprogesterone was high in 46 (59%) patients and low in 14 (17.9%). There were no significant differences between patient subgroups based on 17-hydroxyprogesterone concentration with respect to the time elapsed from the glucocorticoid administration to sample collection. Hormonal concentrations in the three subgroups showed that 11-hydroxyandrostenedione and 11-ketotestosterone were significantly higher in the subgroup with high 17-hydroxyprogesterone compared to the subgroup within target range in both plasma ($p<0.001$ for both 11-hydroxyandrostenedione and 11-ketotestosterone) and saliva ($p<0.001$ for 11-hydroxyandrostenedione and $p=0.001$

for 11-ketotestosterone). Comparing the subgroup with low 17-hydrogyprogesterone against normal 17-hydroxyprogesterone we found that both the 11-oxygenated steroids were significantly lower in the suppressed group, although the difference was more significant in plasma (*p=0.006* for 11-hydroxyandrostenedione and *p=0.005* for 11-ketotestosterone) than in saliva (*p=0.021* for 11-hydroxyandrostenedione and *p=0.043* for 11-ketotestosterone). We found consistent statistical difference between the groups of low and high 17-hydroxyprogesterone for the two 11-oxygenated steroids in both plasma and saliva (*p<0.001*) (**Figures 3 and 4, Supplementary Table 10**) [15]. When comparing age and gender subgroups of patients to their control counterparts in relation to plasma 11-oxygenated androgen concentrations, we found that in most cases control values overlapped with patients who had been classified by plasma 17-hydroxyprogesterone as either suppressed or within target range **(Supplementary Figure 6)** [15].

As a next step, we analyzed the correlations between plasma and salivary 11-ketotestosterone for patient subgroups based on 17-hydroxyprogesterone concentrations. We found strong correlations in all three subgroups (low 17-hydroxyprogesterone group: $r_s=0.792$, $p=0.004$, within target range group: $r_s=0.763$, $p=0.002$ and high 17-hydroxyprogesterone group: $r_s=0915$, $p<0.001$).

Correlations of plasma 11-ketotestosterone with testosterone and 17-hydroxyprogesterone in patients with CAH

In patients with CAH, we found correlations between plasma 11-ketotestosterone and testosterone in the patient group ($r_s=0.691$, $p<0.001$), as well as subgroups of age and gender, with the exception of boys 12-18 years of age ($r_s=0.520$, $p=0.039$). Interestingly, in pubertal boys with 21OHD we found that 11-ketotestosterone and testosterone correlated strongly in saliva ($r_s=0.811$, $p<0.001$). We found strong correlations between plasma 11-ketotestosterone and 17-hydroxyprogesterone for all subgroups of age and gender (**Figure 5**).

Discussion

This is the first study analyzing the correlation of adrenal-specific 11-oxygenated-C19 androgens between plasma and salivary concentrations in children and young people with CAH. It was performed to explore the potential use of measuring the salivary steroid hormones including 11-oxygenated-C19 androgens in assessing disease control in patients with CAH.

Currently, 17-hydroxyprogesterone, androstenedione and testosterone are used for this purpose. However, they all have limitations as indicators of adrenal excess: 17-hydroxyprogesterone concentrations fluctuate widely in relation to the glucocorticoid dose [21], while androstenedione and testosterone are also synthesized by the gonads, which reduces their specificity and relevance as biomarkers of adrenal androgen excess during puberty [18]. Recent studies exploring 11-oxygenated-C19 steroids have proven their adrenal origin and suggested that they can serve as biomarkers of adrenal androgen excess in CAH [13] and women with polycystic ovary syndrome (PCOS) [22]. Biochemical assessment of patients with 21OHD demonstrated increased concentrations of 11-hydroxyandrostenedione, 11-hydroxytestosterone, 11-ketoandrostenedione and 11-ketotestosterone in comparison to healthy controls [13, 14]. Moreover, in adults with CAH 11-oxygenated-C19 androgens were found to correlate with the adrenal volume as marker of adrenal hyperplasia [17]. Our data confirm raised concentrations of 11-hydroxyandrostenedione and 11-ketotestosterone in children and young people with CAH and also suggest that measurement of these adrenal-specific androgens are valuable biomarkers for adrenal androgen excess and disease control in patients with CAH.

Due to the fluctuations in adrenal steroid precursors and hormones in relation to treatment, single measurement of hormonal concentrations is of limited use in CAH and it

was shown that hormonal profiles are needed in order to effectively monitor and adjust glucocorticoid treatment [23]. From this perspective, CAH is similar to type 1 diabetes, where studies involving continuous glucose monitoring have demonstrated better patient outcomes associated with the number of self-managed blood glucose measurements throughout the day [24, 25]. Thus, we are tempted to speculate that there is a need for more suitable tests allowing for more frequent measurement of more specific adrenal steroid hormones in patients with CAH and that such an approach might improve health outcomes if they lead to more frequent adjustments of treatment or more accurate glucocorticoid treatment. Due to their non-invasive nature salivary tests are likely to be more acceptable for patients, allowing for repeated measurements and daily hormonal profiles including classical measured parameters (17-hydroxyprogesterone, androstenedione, testosterone) and 11oxygenated-C19 steroids (11-hydroxyandrostenedione and 11-ketotestosterone).

Our data clearly demonstrate a very tight correlation between plasma and salivary concentrations of these five steroid hormones. Importantly, the best correlations were found for 11-ketotestosterone, an adrenal-derived 11-oxygenated-C19 androgen, as well as 17-hydroxyprogesterone and androstenedione, which are widely used for CAH monitoring [4]. These results were consistent across subgroups of patients based on age and gender. Correlations between plasma and salivary steroid concentrations were also found in controls. However, these correlations in controls were generally not as strong as in CAH patients. This is likely linked to the significantly lower steroid concentrations in controls. However, this appears to be of minor relevance to monitoring of disease control in CAH patients, where raised 17-hydroxyprogesterone concentrations have been suggested as treatment target [4, 26].

The correlations between plasma and salivary steroids observed in our study are generally consistent with the findings of previous research on this topic. Even before the use of LC-MS/MS in hormonal assays, good correlations were found in patients with CAH between salivary and serum 17-hydroxyprogesterone and androstenedione using radioimmunoassays [27]. More recent research conducted on 19 healthy adults measuring plasma and salivary steroid concentrations by LC-MS/MS at four time points during one day showed strong serum to salivary correlations for testosterone, androstenedione, dehydroepiandrosterone and 17-hydroxyprogesterone. The authors also reported diurnal fluctuations of these hormones in saliva, consistent with their fluctuations in plasma [7]. Other studies that focused on measuring salivary 17-hydroxyprogesterone and androstenedione in patients with CAH confirmed correlations between plasma and salivary concentrations for both hormones [9]. Further evidence concluded that using them in combination when monitoring glucocorticoid treatment in patients with 21OHD increased the accuracy of the interpretation [12]. Importantly, another study involving patients with CAH also found a strong correlation between salivary and serum 17-hydroxyprogesterone and described a dynamic response in the salivary 17-hydroxyprogesterone to glucocorticoid treatment [8]. Our findings support this concept as the strength of plasma-salivary correlations was maintained when taking into account the time elapsed between glucocorticoid dose and sample collection. Furthermore, using daily profiles of salivary 17-hydroxyprogesterone was shown to be an effective method in monitoring glucocorticoid treatment in patients with 21OHD [23]. However, no data exist exploring the use of salivary measurements of adrenal-specific 11-oxygenated-C19 steroids as a meaningful test to assess adrenal androgen excess.

We compared patient subgroups based on a previously suggested target range for plasma 17-hydroxyprogesterone [26] in order to explore if 11-ketotestosterone and 11-hydroxyandrostenedione can differentiate between patients who are under- or over-treated with glucocorticoids. A range of 12 to 36 nmol/l for plasma 17-hydroxyprogesterone as previously suggested cutoffs for good control was chosen for this analysis. Admittedly, while

providing a convenient biochemical tool for classifying disease control, 17-hydroxyprogesterone has recognized limitations that challenge the clinical interpretation, especially in relation to low or normal values. This is supported by evidence suggesting that the recommended target range for 17-hydroxyprogesterone results in overtreatment [2]. Moreover, we acknowledge that our results were based on hormones measurements conducted at variable times after the morning glucocorticoid dose, which further limits the clinical relevance of plasma 17-hydroxyprogesterone concentrations. Nevertheless, the strong correlations between plasma and salivary 11-ketotestosterone and 11-hydroxyandrostenedione in the subgroups with low, normal and high 17-hydroxyprogesterone are promising with regards to the potential as marker of treatment control. Importantly, we found consistent overlapping of both plasma and salivary concentrations for 11-ketotestosterone and 11-hydroxyandrostenedione between these subgroups, despite significant differences in the overall concentrations expressed by median with interquartile range. This finding might well be attributable to the limited value of 17-hydroxyprogesterone as marker of treatment control when not taking strictly timed samples, which is difficult to achieve in clinical practice. The widespread individual variation of 17-hydroxyprogesterone concentration in relation to the time of the day and the glucocorticoid dose is well-established, the clinical parameters remaining the gold standard for adequate control in practice [2]. In our clinical practice the relevance of 17-hydroxyprogesterone as a biochemical marker is limited to highlighting over-treatment, while androstenedione and testosterone are better biomarkers for under-treatment. Moreover, we acknowledge that in our study the time of sample collection in relation to the glucocorticoid dose varied among patients, further limiting the reliability of androgens concentrations in establishing treatment control. Such reflections emphasize the need to identify more reliable biochemical markers of disease control that are less prone to fluctuations.

Although overall the plasma concentrations of 17-hydroxyprogesterone, androstenedione, 11-hydroxyandrostenedione and 11-ketotestosterone were significantly higher in patients compared to controls, when analyzing the scatterplots for the distribution of individual values, we identified consistent overlap between patients and controls. This was especially pronounced in the case of the two 11-oxygenated androgens and much less noticeable for 17-hydroxyprogesterone. On closer examination we found that in the majority of cases the overlap with control values corresponded to patients identified to have the plasma 17-hydroxyprogesterone either in the target or the suppressed range. This finding supports the potential clinical usefulness of 11-oxygenated androgens in defining undertreatment in patients with CAH. In addition, this further highlights a need to employ hormonal profiles of several steroid hormones as reliable biochemical monitoring for assessing disease control.

Whilst for the majority of measured hormones, higher plasma concentrations were detected in patients, this was not the case for testosterone. Plasma testosterone was significantly higher in male controls compared to male patients. In contrast, girls and children of prepubertal age had significantly higher testosterone concentrations compared to their controls. This is very likely due to the dual origin of testosterone from adrenals and gonads, with a physiological rise in healthy pubertal males. In male patients with 21OHD, the suppression of testicular-derived testosterone by adrenal androgen excess explains the differences noted between patients and controls and confirms observations in adult males with CAH [18, 28]. The increased concentration of testosterone in females and prepubertal individuals is likely linked to adrenal androgen excess. By contrast, 11-ketotestosterone plasma and salivary concentrations were significantly higher for all patient subgroups. These findings are consistent with those reported by other studies that explored androgen profiles in patients with CAH [13, 14], supporting the notion that 11-ketotestosterone is a superior marker of adrenal androgen excess in CAH. Of note, we found that in the overall group of

CAH patients, 11-ketotestosterone correlated with testosterone in both plasma and saliva with the correlation being consistently stronger in saliva. In pubertal boys we found no correlation between plasma 11-ketotestosterone and testosterone in patients and controls. Another recent study demonstrated negative correlation between 11-ketotestosterone and testosterone in pubertal males and positive correlation in pubertal females, attributing these findings to the suppression of testicular testosterone synthesis by adrenal androgen excess in 21OHD [13]. Interestingly, in the subgroup of pubertal male patients, we found strong correlations between testosterone and 11-ketotestosterone concentrations in saliva, despite the absence of a correlation in plasma. This suggests that salivary testosterone is a poor indicator of plasma testosterone, which has been suggested in previous studies [29, 30]. Possible explanations for the discrepancies between plasma and salivary testosterone relate to the different protein content between plasma and saliva impacting on the binding and availability of testosterone [29], as well as the conversion of testosterone to androstenedione in the salivary glands [31]. Moreover, in interpreting our results consideration must be given to the risk of measurement errors related to sample storage that was reported for testosterone when analyzed by LC-MS/MS [19].

Our findings related to the correlation between plasma cortisol and salivary cortisone concentrations are consistent with recently published evidence [21, 32] demonstrating that salivary cortisone provides a good reflection of plasma cortisol. By contrast, we found salivary cortisol to be very high for a significant number of patients, which limited its usefulness as marker for plasma cortisol. We believe that the most likely explanation for the dramatically raised salivary cortisol was sample contamination following administration of oral hydrocortisone. Applying our findings to clinical practice, we would recommend that salivary cortisol should be collected prior to the glucocorticoid dose, while samples taken randomly or after the glucocorticoid dose should be tested for both cortisone and cortisol concentrations, to allow differentiation between overexposure to cortisol and sample contamination. Interestingly, while in controls we found consistent correlations between plasma cortisol/salivary cortisone and the five adrenal steroids measured in plasma and saliva, this was not the case in the CAH group, even when controlling for the sample collection time and excluding the cases of excessively high salivary cortisol. Previous research exploring the pharmacokinetics of oral cortisol in children with CAH in relation to 17-hydroxyprogesterone and androstenedione profiles yielded contradictory results. An earlier study using RIA for the steroid assays found a negative correlation between plasma cortisol and 17-hydroxyprogetsreone, but not androstenedione, in 19 patients with non-suppressed 17-hydroxyprogetserone [33]. However, a more recent study conducted on 34 children with CAH using LC-MS/MS for hormone measurements did not identify a relationship between neither 17-hydroxyprogesterone nor androstenedione and cortisol [34]. The authors attributed their results to the significant interindividual variability in cortisol pharmacokinetics and in the response of the hypothalamo-pituitary-adrenal axis to treatment.

A limiting factor in our study consisted of a significant number of insufficient/unsuitable salivary samples, which in turn reduced the number of paired samples available for correlations to 65 for patients and 58 for controls. We also acknowledge that our study involved single time-point hormonal measurements and did not explore the aspect of salivary hormonal variations throughout the day. Moreover, the recommendation of the Endocrine Society for monitoring treatment in CAH is that hormonal measurements should be timed in relation to medication [2]. This was not feasible in our study as the timing of glucocorticoid doses varied widely among participants; thus, our results were based on random hormonal measurements, limiting their clinical relevance. However, there is evidence indicating that in patients with CAH adrenal androgen secretion follows a circadian rhythm that is influenced by glucocorticoid medication [21]. In our study, although there was no standardization of

sample collection in relation to the glucocorticoid dose, the plasma and saliva specimens were obtained between 8:00 and 9:00 am when androgen concentrations are expected to be higher. A study conducted on healthy adults undergoing the Synacthen® test identified a 60 minutes lag in the rise of salivary concentrations compared to plasma concentrations for cortisol and cortisone [32]. This finding highlights another important aspect namely, that the correlation between salivary and plasma androgen hormones may be time sensitive. Thus, further research exploring the fluctuations of salivary androgens in relation to replacement therapy may yield more accurate information regarding their potential use in monitoring therapy control.

In summary, we have demonstrated a strong correlation between plasma and salivary concentrations of androgen hormones in patients with CAH. To our knowledge, this is the first study to demonstrate correlations between plasma and salivary concentrations for 11-oxygenated-C19 androgens in children with CAH. Our findings suggest that the combination of salivary steroid hormones can serve as a non-invasive monitoring tool in CAH, which will provide a significant amount of additional information and will ultimately improve management and outcomes in CAH.

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Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure 1. Comparison of plasma steroid concentrations between patients (dark dots) and controls (clear dots). The left column represents the results obtained for boys younger than 12 years of age (left pair of scatter columns) and 12 to 18 years of age (right pair of scatter columns) for 17-hydroxyprogesterone (A), androstenedione (B), testosterone (C), 11-hydroxyandrostenedione (D) and 11-ketotestosterone (E). The right column represents the results obtained for girls younger than 12 years of age (left pair of scatter columns) and 12 to 18 years of age (right pair of scatter columns) for 17-hydroxyprogesterone (F), androstenedione (G), testosterone (H), 11-hydroxyandrostenedione (I) and 11-ketotestosterone (J). The horizontal bars within the scatter columns correspond to the median and interquartile range. Statistical analysis was performed by Mann Whitney U test. (17OHP: 17-hydroxyprogesteone, A4: androstenedione, T: testosterone, 11OHA4: 11-hydroxyandrostenedione, 11KT: 11-ketotestosterone)

Figure 2. The scatter graphs show the relation between plasma (vertical axis) and salivary (horizontal axis) steroid concentrations in patients with CAH for 17-hydroxyprogesterone (A), androstenedione (B), testosterone (C), 11-hydroxyandrostenedione (D) and 11-ketotestosterone (E). The upper left corner of each scatter graph depicts the results of the Spearman correlations (r_s and p value). (17OHP: 17-hydroxyprogesteone, A4: androstenedione, T: testosterone, 11OHA4: 11-hydroxyandrostenedione, 11KT: 11-ketotestosterone)

Figure 3. Comparison of plasma steroid concentrations between patient subgroups based on plasma 17-hydroxyprogesterone concentrations: high (dark dots), normal (clear dots) and low (dark triangles) for androstenedione (A), testosterone (B), 11-hydroxyandrostenedione (C) and 11-ketotestosterone (D). The horizontal bars within the scatter columns correspond to the median and interquartile range. The p values are the results of the Mann Whitney U test. (17OHP: 17-hydroxyprogesterone, A4: androstenedione, T: testosterone, 11OHA4: 11-hydroxyandrostenedione, 11KT: 11-ketotestosterone)

Figure 4. Comparison of salivary steroid concentrations between patient subgroups classified based on plasma 17-hydroxyprogesterone concentrations: high (dark dots), normal (clear dots) and low (dark triangles) for 17-hydroxyprogesterone (A), androstenedione (B), testosterone (C), 11-hydroxyandrostenedione (D) and 11-ketotestosterone (E). The horizontal bars within the scatter columns correspond to the median and interquartile range. The p values are derived from the analysis by Mann Whitney U test. (17OHP: 17-hydroxyprogesterone, A4: androstenedione, T: testosterone, 11OHA4: 11-hydroxyandrostenedione, 11KT: 11-ketotestosterone)

Figure 5. The scatter graphs in the left column present the relation between plasma 11-ketotestosterone (vertical axis) and plasma testosterone (horizontal axis) in boys less than 12

years of age (A), boys of 12 to 18 years of age (B), girls less than 12 years of age (C), girls of 12 to 18 years of age (D). The scatter graphs in the right column present the relation between plasma 11-ketotestosterone (vertical axis) and plasma 17-hydroxyprogesterone (horizontal axis) in boys younger than 12 years of age (E), boys between 12 to 18 years of age (F), girls younger than 12 years of age (G), girls between 12 to 18 years of age (H). The results of the Spearman correlations (r_s and p value) are shown in in the upper left corner of each scatter plot. (11KT: 11-ketotestosterone, T: testosterone, 17OHP: 17-hydroxyprogesteone)

Table 1. Group characteristics. The age is expressed as median with interquartile range. The statistical difference was explored by the Chi-squared test.

	Patients	Controls	Statistical difference
Number	78	62	
Age (years)	12.8(10.4-15.6)	13.3(10.5-16.3)	p=0.36
< 12 years	35(44.9%)	27(43.5%)	p=0.87
12-18 years	43(55.1%)	35(56.5%)	
Girls	43(55.1%)	36(58.1%)	p=0.72
Boys	35(44.9%)	26(41.9%)	
Tanner stage			p=0.008
1-2	17(21.7%)	25(40.3%)	
3-4	33(42.3%)	29(46.7%)	
5	22(28.2%)	5(8%)	
Not known	6(7.6%)	3(4.8%)